International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.2, pp 669-676, April-June 2012

Development and Validation of UV Spectrophotometric and HPLC Methods for Quantitative Determination of Chloroquine and Amodiaquine in Pharmaceutical Formulations

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Abstract: A rapid and sensitive reverse phase HPLC method with UV detection and UV spectrophotometric methods for the analysis of chloroquine (CQ) and amodiaquine (AMQ) in formulations were developed. Chromatography was performed using external standard method with mobile phase containing a mixture of phosphate buffer (pH 6.60) and acetonitrile (40:60 v/v) for CQ and phosphate buffer (pH 3.50): acetonitrile (45:55 v/v) for AMQ respectively. The samples were injected onto Eclipse, XDB C_{18} (150 mm x 4.6 mm, 5 µm) column. The flow rate was 1.500 ml/min. The samples were detected at 330 nm and 345 nm for CQ and AMQ respectively. The assay was linear in range from 30 to 150 $\mu g.ml^{-1}$ with a correlation coefficient (r = 0.9963 and 0.9802 for CQ and AMQ) respectively. The spectrophotometric method was performed at 410.05 nm, using ion-pair extraction procedure. The linearity demonstrated a correlation coefficient of 0.9560 and 0.95008 for CQ and AMQ respectively. The methods were validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and robustness as per ICH and USP guidelines. The proposed methods were successfully applied in determination of these drugs in formulations.
Key words: *Chloroquine, amodiaquine, UV spectrophotometer and HPLC.*

INTRODUCTION

Malaria is still one of the most severe infectious diseases globally which is widespread mainly in the tropical and subtropical regions. It kills more people each year than any other infectious diseases except AIDS and tuberculosis [(1)]. Although it is difficult to obtain an exact figure of the malaria cases, the World Health Organization (WHO) estimates that malaria is responsible for over 300 million clinical cases and over one million deaths annually. About 40% of the global population is estimated to be at risk. Malaria is not just a disease commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development [(1,2)].

Chloroquine phosphate is (RS) – 4 – (7 – chloro – 4 – quinolylamino) pentyldiethylamine diorthophosphate. It contains not less than 98.5% and not more than 101.0% of $C_{18}H_{26}ClN_3$, $2H_3PO_4$, calculated with reference to the anhydrous substances BP [(3)]. Chloroquine is a 4 – aminoquinoline which has marked and rapid schizontocidal activity against all infections of *P. malariae* and *P. ovale* and against

chloroquine-sensitive infections of *P. falciparum* and *P. vivax* [4]. It is also gametocytocidal against *P. Vivax, P. malariae,* and *P. ovale* as well as immature gametocytes of *P. falciparum* and it is not active against intrahepatic form [5].

Amodiaquine is synthesized from 4, 7-dichloroquinoline and 4-acetamido- diethylamino-o-cresol, can also alternative synthesis from 2-aminomethyl-p-aminophenol and 4, 7-dichloroquinoline. Amodiaquine is a 4-aminoquinoline antimalarial drug similar in structure and activity to chloroquine. Like chloroquine, it also possesses anti-pyretic and anti-inflammatory effects [6]. In a systematic review of relevant studies conducted over the past ten years in Africa for the treatment of uncomplicated *falciparum* malaria, amodiaquine proved significantly more effective than chloroquine in clearing parasites and with a tendency also for faster clinical recovery. This difference was also observed in areas with considerable chloroquine resistance [7].

Several different physical and chemical tests can be used for the analysis of antimalarials. There are a variety of chemical tests available which ranged from testing for the presence of active ingredient in a tablet to quantifying the amount of antimalarial present in the bloodstream [8, 9]. The tests can also vary in complexity ranging from simple field tests to advanced analytical techniques. Where high technical laboratory equipment may be unavailable, some field tests may include thin-layer chromatography (TLC) and colorimetry [8, 9]. Information on the type of counterfeiting and the drug's origin can only be determined with more advanced techniques [9]. The aim of this work is to develop simple spectrophotometric and RP-HPLC methods for the estimation of these antimalarials in dosage form.

MATERIALS AND METHODS

MATERIALS

Equipments

The chromatographic equipment consisted of a series 1100 HPLC system with manual injector, variable wavelength detector (VWD) and Vacuum degasser (Agilent Technologies Deutschland GmbH, Waldron, Germany). An Eclipse C_{18} (Agilent, Germany) column (150mm x 4.6mm, 5µm particle size) was used for the separation.

The spectroscopic equipment consisted of a UV/VIS 1650PC (Shimadzu, Japan); with 2 mm slit width and 1 cm cuvette sample holder was used.

Chemicals and Reagents

Potassium dihydrogen phosphate (BDH), phosphoric acid (BDH), Sodium perchlorate salt (Analar), methanol (99.9%; HPLC-grade) and Acetonitrile (99.9%) were purchased from Sigma Aldrich Germany. Pure Amodiaquin and Chloroquine phosphate salts (Sigma Aldrich, USA), Carbonate Buffer (50mM) pH 9.5±0.1, Bromo-thymol blue solution (0.01 mol/l) and Dichloromethane (Analar). Tablets from different brands were procure from local pharmacies and chemists'

METHODS

HPLC Analysis

HPLC Conditions for Chloroquine diphosphate Analysis:

Mobile phase: Phosphate Buffer: Acetonitrile (40:60v/v), the pH of the phosphate buffer was adjusted to 6.60 with sodium perchlorate

Flow rate: 1.500mL/min.

Column: Eclipse, XDB-C_{18} (150mm, 4.6mm, 5µm).

Detection wavelength: 330nm

HPLC Conditions for Amodiaquine hydrochloride Analysis:

Mobile phase:Phosphate Buffer: Methanol (45:55v/v), the pH of the phosphate buffer was adjusted to 3.5 with perchloric acid.

Flow rate:1.50mL/min.

Column:Eclipse, XDB-C_{18} (150mm, 4.6mm, 5µm).

Detection wavelength: 345nm

Spectrophotometric Analysis

Preparation of Standards/Chemical Used:

a) Chloroquine diphosphate and amodiaquine dihydrochloride (standards) were prepared by dissolving 120 mg of pure samples in 400 ml of distilled water to give the concentration of 300 mg/L from which serial dilutions were made from 7.5 - 120 mg/L.
b) Carbonate buffer was prepared by mixing solution of $KHCO_3$ (50 g/500 ml) and Na_2CO_3 (50.4g/500 ml) in the ratio of 4:1 by volume; pH adjusted to 9.5 by adding drop wise disodium carbonate and monitored using pH meter.
c) Bromo-thymol blue was prepared by dissolving 6.24 g of powdered form of the salt in aqueous solution to make the required concentration. From this concentration working solution was prepared by diluting 3.25 ml of the stock with 46.85 ml of carbonate buffer. (The solution is stable over four weeks at 35^0C).

Determination of λmax for Chloroquine and Amodiaquine-bromo-thymol Complexes

Exactly 2 ml each of 25 mg/L chloroquine and amodiaquine were transferred into a 10 ml test tubes, 2 ml of bromo-thymol blue solution and 3 ml of dichloromethane were added. The mixture was shaken for approximately 30 seconds and the mixture was allowed to stand for 30 minutes. The mixture was separated into two phases, the upper layer discarded and 1.0 ml of distilled water and 2.0 ml of bromo-thymol blue were added again. The mixture was shaken for 30 seconds, the upper layer discarded. The complex colour formed was scanned using UV/Visible spectrophotometer to determine the λmax. The λmax was found to be 410.05 nm and it was used for the analysis.

Preparation of Calibration Curves for Chloroquine and Amodiaquine Standards

Serial dilutions (7.5, 15, 30, 60, 90 and 120 mg/l) were made from the stock solutions each of chloroquine and amodiaquine. Exactly 2 ml of each serially diluted solution was transferred into 10 ml test tubes. The rest of the procedure followed as in case of determination of λmax above against a sample blank.

Analysis of Tablets in Formulation (Extraction)

Twenty tablets of each drugs sampled were weighed accurately and finely powdered using mortar and pestle. A quantity of powder equivalent to an average of weight of single tablet was dissolved in distilled H_2O. The mixture was filtered using 0.2 µm filter paper and allowed to stand for 30 minutes.

Determination of Chloroquine and Amodiaquine in Tablets Sample

About 2 ml of the filtered solutions from extracted aliquots each of chloroquine and amodiaquine were transferred into 10 ml test tubes, 2 ml of bromo-thymol blue solution and 3 ml of dichloromethane were added. The mixture was shaken for approximately 30 seconds and the mixture was allowed to stand for 30 minutes. The mixture separated into two phases the upper layer discarded, and 1.0 ml of distilled water and 2.0 ml of bromo-thymol blue were added again. After being shaken for 30 seconds the upper layer discarded. The absorbance was then measured using Shimadzu UV-visible spectrophotometer at λmax = 410.05 nm in triplicates and their respective concentration determined from the calibration curves already established.

Validation of the Methods

Validation of the optimized HPLC and spectrophotometric methods were carried out with respect to the following parameters.

Linearity and Range

From chloroquine and amodiaquine standards stock solutions, aliquots of 5, 10, 20, 30, 40 and 50 µg/mL concentrations were transferred to 10 mL volumetric flask and the volume were made up to the mark with mobile phase to obtained concentration of 5-50 µg/mL. The solution of 20 µL was injected onto the column with the help of Hamilton syringe. All measurements were repeated three times for each concentration. The calibration curves were established using mean peak area Vs concentrations of standard drugs. So also for the spectrophotometric method where mean absorbance was plotted against the respective concentration of the standards.

Precision

The precision (intermediate) of the methods were verified by repeatability, intraday and interday precisions. Repeatability studies were performed by analysis of three different concentrations of the drugs, six times on the same day. Intraday precision was determined by analyzing the five different concentrations of the standards sample in triplicates at different time intervals on the same day and while on different day for interday precision given concentration was analysed in triplicate.

Limits of Detection and Quantification

The LOD and LOQ were determined separately based on the calibration curves. The Standard Deviation of their intercept and slope of the regression line were used. The LOD and LOQ were calculated using the formulae,

$$LOQ = 10 \times \delta/S$$

$$LOD = 3.3 \times \delta/S$$

Where,

S is the slope of regression line**.**

δ = standard deviation of y-intercept on the regression line

Robustness of the methods

To evaluate the robustness of the developed method, minute variations in the methods parameters were done. The parameters such as, effect of change in pH of mobile phase, flow rate, effects of mobile phase ratio on the retention time were determined.

Table1 (a): Intra-day Precisions

Concentration (µg/mL)	**CQ** RSD (%)	**AMQ** RSD (%)
10	43.1	80.7
12.5	24.3	71.9
25	97.1	46.8
50	35.8	30.9
100	22.3	63.9

RSD is relative Standard Deviation

Table1 (b): Inter-day Precisions

Day	**CQ** RSD (%)	**AMQ** RSD (%)
1	50.2	43.9
2	48.2	53.7
3	48.8	59.3
4	49.9	50.5
5	51.7	52.3

RSD relative standard deviation

Table2 (a): Repeated Measurements (Intra-day precision)

No of Runs	**Concentration (µg/mL)**
1	95.9
2	99.5
3	98.8
4	99.1
5	98.9
6	97.0
7	97.4
8	96.8
9	95.9
10	95.5

Table2 (b): Repeated Measurements on Two Different Occasions (Inter-day precision)

No of Runs	1st Occasion Concentration (µg/mL)	2nd Occasion Concentration (µg/mL)
1	99.1	98.2
2	98.1	97.7
3	99.5	99.1
4	99.5	99.0
5	98.4	99.6
6	97.5	98.8
7	97.5	98.3
8	96.9	97.6
9	97.7	98.7
10	98.9	99.1

Table3 (a): LOD and LOQ for Spectrophotometric Method

Parameters	Chloroquine	Amodiaquine
Limit of detection (mg L^{-1})	3.60	22.8
Limit of quantification (mg L^{-1})	112.8	714.7

Table 3(b): LOD and LOQ for CQ and AMQ Using HPLC

Parameters	Chloroquine	Amodiaquine
Limit of detection (µg mL^{-1})	25.5	140.2
Limit of quantification (µg mL^{-1})	247.8	431.5

Table 4(a): Percent Content of Chloroquine Tablets by Ion-pair Extraction Method

Sample	Concentration (w/w)	%Content
CQ1	177.0	70.8
CQ2	178.9	71.5
CQ3	180.7	72.3
CQ4	181.6	72.6
CQ5	182.3	72.9

*CQ Chloroquine

Table 4(b): Percent Content of Amodiaquine Tablets by Ion-pair Extraction Method

Sample	Concentration (w/w)	%Content
AMQ1	262.4	104
AMQ2	221.3	88.5
AMQ3	238.0	95.2

Table 5(a): Percent Content of Some Selected Amodiaquine Tablets by HPLC Method

Sample	Concentration (w/w)	%Content
AMQ1	50.1	19.9
AMQ2	49.3	20.2
AMQ3	52.2	19.2

*AMQ Amodiaquine

Table 5(b): Percent Content of Some Selected Chloroquine Tablets by HPLC Method

Sample	Concentration (w/w)	%Content
CQ1	3.98	33.2
CQ2	3.60	30.0
CQ3	4.17	34.7

CQ Chloroquine Tablet

RESULTS

The results obtained for the validation of these methods for chloroquine and amodiaquine in current study involving methanol, acetonitrile and phosphate buffer for HPLC and dichloromethane, bromo-thymol blue for spectrophotometry are given below.

Linearity

The drugs response using spectroscopic method were (r^2 = 0.9613 for CQ, and 0.9847 for AMQ) over the concentration range between 25 – 400 mg/L. The mean (±SD) value of the slope, intercept and correlation coefficient for CQ and AMQ were 0.0055(±0.17), 0.0004(±0.10), 0.9613(±0.15) and 0.009(±0.045), 0.00004(±0.010), 0.9847(±0.016) respectively. The HPLC method shows r^2 = 0.994 for CQ and 0.9554 for AMQ over the range of 10 - 50µg/mL.

Precision

The results of the repeatability, intra-day and inter-day precisions for spectrophotometric methods are shown in Table 1(a) and Table 1(b) while for the HPLC method are shown in Table 2(a) and Table 2(b) respectively. The developed methods were found to be precise as RSD values for repeatability of intra-day and inter-day precisions were < 10%.

LOD and LOQ

The LOD and LOQ were separately determined based on the calibration curves for the methods developed. The LOD and LOQ for spectroscopic methods were 3.6, 22.8 mg/L and 112.8, 714.7 mg/L for CQ and AMQ respectively for HPLC method the LOD were 25.5, and 140.2µg/mL and LOQ were 247.8 and 431.5µg/mL for CQ and AMQ presented in tables 3a and 3b respectively.

Analysis of Drugs in Formulations

The drugs sample were analysed the same way as the reference standards and their concentrations determine from the established standard curves and their percent content were presented in tables 4a, 4b, 4c, 5a and 5b respectively.

DISCUSSION

Quantitative analysis using UV/VIS spectrophotometer is based on development of calibration curves using standard samples. Once a standard curve is successfully developed, the concentration of the sample can either be extrapolated or interpolated. The standard curves for the chloroquine and amodiaquine were determined using a suggested [10] in determining these antimalarials in biological fluids but we used the principles to determine these antimalarials in aqueous solution. The procedure was tested with or without any serious modification to these antimalarials and it showed relatively low correlation coefficients; 0.9613 and 0.9847, respectively this finding agreed with the findings [10] where biological fluids was used to determined these antimalarial drugs and the result obtained was r^2 = 0.953. However, the r^2 values fall within the acceptable range set [11, 12] (r^2 = 0.95 – 0.999) and hence they are still acceptable.

HPLC has a number of applications amongst which are; preparative, chemical separation, purification, identification and quantification. Quantitative analysis using HPLC is based on development of calibration curve using standard sample. The development involves plotting either the concentration against the mean peak areas or mean peak height. According to the theory, both the mean peak areas and mean peak heights are proportional to the respective concentrations of a given analytes.

The λ_{max} for quantitative analysis was first determined by dissolving small amount of standard samples in one of the mobile phase to be used and scanned within the UV/VIS region to obtain the maximum absorption at a particular wavelength which serves as λ_{max} for that analyte. From the results obtained CQ, AMQ have the λ_{max}; 330 nm, and 345 nm respectively which were used for quantitative analysis of these compounds.

The results obtained for calibration curves show correlation coefficients of 0.996, 0.980 for CQ, and AMQ, respectively [11, 12].

Determination of HPLC Method Performance

Validation is the post-development process whereby methods developed are tested for some parameters. The most important parameters to be determined are; precision, LOD, LOQ and robustness. To determine precision in this case, a certain concentration (specific) is to be prepared in duplicates and injected into the column ten times separately from each other and different intervals say about 2 hours and how close the findings are, that show how precise the method is to detect the analytes for that given concentration. To determine precision for CQ and AMQ, concentration of about 100µg/mL and 200 µg/mL were used and injection into the column were made in separate occasion, ten times with two replicates (prepared differently), the results obtained and presented (Tables 2(a), 2(b)) show that the values are relatively the same but some differences were observed; these could be due to the time allowed for the machine to equilibrate and establish constant base line between two injections i.e. time intervals. These are essentials because if not well established, the detector may receive false signal due to carry-over analyte remaining in the mobile phase.

LOD and LOQ were calculated from the standard deviation and slope of the calibration curves. From the calibration curves, LOD were found to be 140.2µg/mL, 25.5µg/mL for AMQ, and CQ, respectively while the LOQ were 431.5µg/mL, 247.8µg/mL for AMQ, and CQ. These show that the method for CQ is sensitive and can quantify CQ from 25.5µg/mL and above followed by that of AMQ. No specific amounts or range were given for LOD and LOQ by any of the standard organizations or procedures like that of USP and ICH guidelines but the lower the values for LOD the higher the sensitivity of the to the analytes [11, 12].

Method Optimisation

The proper combination of mobile phase was all-important for successful liquid chromatography (David, 1999). In this work, the first mobile phase combinations for chloroquine and amodiaquine, chloroquine as internal standard was methanol/acetonitrile in (50:50 v/v) but was unable to separate the compounds successfully. The composition of the mobile phase was changed to (75:25 v/v) methanol/acetonitrile in order to separate the two compounds but the result was the compounds were eluted with fused peaks. Good elution of the two peaks was not achieved after repeated attempt on different compositions of methanol and acetonitrile. But after substituting methanol with phosphate buffer (3.0) and acetonitrile (85:15 v/v) at pH of 7.3 the analytes AMQ and CQ were clearly resolved. Further adjustment of the two mobile phases i.e. acetonitrile and phosphate buffer in (45:55 v/v) proportions was used for quantitative analyses of both amodiaquine and chloroquine because it gave good resolution of peaks.

Tests for Robustness

The robustness of HPLC methods for assaying these antimalarials was tested during the method development. The parameters observed were pH, retention time, resolution of peak and flow rate etc. It was observed that these parameters have serious consequence on any HPLC method developed. The pH-values of the mobile phases affect the ionization of drugs especially when internal standard is considered. This is linked to resolution of peaks and the retention time. The pH-value of the mobile phases after mixing is the most important; drugs tend to resolve clearly when the mobile phase pH-values, after mixing, do not exceed their pKa's values [10]. Therefore, different pH values were tested until the optimum values were obtained. At such pH the drugs were clearly resolved. The effect of changed in pH was tested when aqueous phosphate buffer with pH 2.8, 4.3, 5.64 and 6.61 was mixed with methanol in 3:1 v/v. The pH of the mobile phases changes to 4.13, 5.30, 7.10 and 7.96, respectively. This is because methanol is protonic, while acetonitrile is aprotonic. This will affect chloroquine and amodiaquine because, at pH 9.5, both chloroquine and amodiaquine will exist predominantly in ionic form [10]. The pKa's values of each were 8.4 and 10.8 for CQ and AMQ has 8.14 and 7.08. The reason for these observed pKa's especially for CQ and AMQ is that AMQ is a weaker base than CQ [14].

Retention time is greatly affected by mobile phase combinations and flow rate. It was observed that during method development to achieve good resolution of peaks and good symmetry of peaks different combinations. Mobile phase tested were taking into consideration the effect of pH on the ionization of drugs which will affect their resolution and consequently retention time. This was mostly observed if internal standard method is in consideration. Good selection of mobile phase combination may shorten the retention time but mostly and importantly, for good resolution of peaks to be achieved, retention time has to be sacrifice. The observation made here is that the method is affected by changes in pH, mobile phase combinations, flow rate and pressure. Therefore, test of robustness in any method is essential for that method to be tested in other laboratories successfully.

Spectrophotometric Method Validation

After the method was successfully developed there was need to validate the methods to determine the level of precision. Precision determination is divided into two stages according to the ICH guidelines. First is the determination of intra-day precision (repeatability) and inter-day precision (intermediate). These precisions are important in that it show how precised the method is and its reproducibility within and after some days and how accurate the method is expected at a given concentration.

The results for intra-day precision for CQ and AMQ are presented in Table 1(a) which shows that CQ has 97.1% precision when compared with that of AMQ at the same level of concentration (25µg/mL) with 46.8%. These precisions indicate that the method is sensitive and reproducible for CQ than for amodiaquine.

After intra-day precision determinations, the methods undergo another precision test after some days to ascertain the methods for intermediate precision (inter-day) presented in Tables 1(b). From the results obtained it show a relatively uniform precision of 50.2 – 51.7% for CQ, 50.5 – 53.7% AMQ, at different levels of concentrations. These findings agreed with the findings of Bergqvist *et al.* (1985) [10].

HPLC and Spectrophotometric Analyses of Chloroquine and Amodiaquine Tablets

In post-developmental and validation processes using HPLC, some drugs were sampled and analysed. The concentrations of the drugs were interpolated from the standard curves. The result obtained show that the percent content of API of these drugs were 19.9, 20.2 and 19.2% for AMQ1, AMQ2 and AMQ3, respectively. For CQ, the percent contents were 33.2, 30.0, 34.7 and 84.0% for CQ1, CQ2, CQ3 and CQI; with CQI, which is chloroquine injection having highest percent content of API when compared with the tablets. These values of percent contents for both CQ and AMQ when compared with percent content of standard method [11, 12], i.e. 95 – 105% are very small this could be due to the extraction procedure before the analysis. Another reason could be due the effect of pH change on the analytes; they tend to ionize and that could actually results in possible interaction more with stationary phase than the mobile phase. In a nutshell, the percent content is below the acceptable limit. This could suggest the need for more evaluation of the extraction procedure and possibly the mobile phase combinations.

The results obtained from spectroscopic method and presented in Table 3(a) show that the method can detect and quantify the drugs in formulations and percent content of their API were calculated. The method show appreciable percent content for CQ with 70.8 – 72.9%, CQ5 contains more API (72.9%) when compared with CQ1 (70.8%). This variation in API could be due to the quality of the drugs or procedure used for extraction. While the percent content for AMQ1 (104%) and AMQ3 (95.2%) are high when compared with AMQ1 (88.5%). But the results from AMQ drugs sample can be compared with that of standard method [11, 12].

CONCLUSION

HPLC with UV detection and Spectrophotometric methods for the analysis of chloroquine, and amodiaquine in formulation has been developed, which takes reasonable time to complete. The spectrophotometric method was based on the ion-pair extraction using bromo-thymol blue as counter ion and dichloromethane. Based on the results obtained we can conclude that; the methods are selective and sensitive for all the analytes, and thus, the method are applicable to these drugs in formulations.

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